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# Seed Germination and Root Elongation Toxicity Tests in Hazardous Waste Site Evaluation: Methods Development and Applications

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Seed germination tests measure soil toxicity directly, while root elongation tests consider the indirect effects of water-soluble constituents which may be present in site-samples. In the seed germination toxicity test, site-soil is mixed with a reference soil to yield exposure concentrations into which test seeds are planted. Germination is evaluated after a five-day exposure, and effective concentrations associated with a 50% reduction in seed germination are calculated. Root elongation tests evaluate site-soil eluates. Here, seeds are placed onto moistened filter paper which lines Petri dish exposure chambers; exposure chambers are then covered and incubated in complete darkness for five days. Inhibition of root elongation is calculated as an $EC_{50}$ (exposure concentration which yields a 50% reduction in root length relative to controls) upon termination of the test.

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**SEED GERMINATION AND ROOT ELONGATION TOXICITY TESTS IN HAZARDOUS WASTE SITE EVALUATION: METHODS DEVELOPMENT AND APPLICATIONS,** Greg Linder[1], Joseph C. Greene[2], Hilman Ratsch[2], Julius Nwosu[1], Sheila Smith[1], and David Wilborn[1], NSI Technology Services Corporation[1], U.S. Environmental Protection Agency[2], Environmental Research Laboratory, 200 S.W. 35th Street, Corvallis, OR.

**ABSTRACT:** Techniques modified from methods originally developed in the plant and weed science disciplines have yielded short-term tests which assess toxic chemical effects on plants. The seed germination and root elongation bioassays are laboratory toxicity tests which have evolved to assess the direct and indirect toxicity of hazardous waste site-soils. These assessment tools are part of a collection of single-species toxicity tests which evaluate toxicity endpoints (seed germination and root elongation) pertinent to ecological assessments for terrestrial hazardous waste sites. Seed germination tests measure toxicity associated with soils directly, while root elongation tests consider the indirect effects of water-soluble constituents which may be present in site-samples. In the seed germination toxicity test, site-soil is mixed with a reference soil to yield a logarithmic series of exposure concentrations into which test seeds are planted. Germination is evaluated after a five-day exposure, and effective concentrations associated with a 50% reduction in seed germination are calculated. Contrasted to this direct test of soil toxicity, the root elongation test evaluates soil eluates which are prepared from site-samples and contain water-soluble soil constituents potentially available to plants on-site and off-site. For the root elongation test, seeds are placed onto moistened filter paper which lines Petri dish exposure chambers. Then, the exposure chambers are covered and incubated in complete darkness for five days; inhibition of root elongation is calculated as an $EC_{50}$ (exposure concentration which

yields a 50% reduction in root length relative to controls) upon termination of the test. By using a variety of plant species and developing a comparative toxicity data base, both seed germination and root elongation toxicity tests may be applied on a site-specific basis and contribute to the toxicity assessment required as part of an ecological assessment for a hazardous waste site.

KEY WORDS: seed germination, root elongation, terrestrial toxicity testing, ecological assessment, toxicity assessment, hazardous waste site remediation

## Introduction

Plants are recognized as critical elements of terrestrial ecosystems and must be considered as part of any ecological hazard assessment [1, 2]. Ecological hazard assessment requires interrelated evaluations of toxicity and exposure. While assessment methods are less well developed for terrestrial sites [3], the evaluation of ecological effects in these systems requires methods not unlike those frequently outlined for hazard evaluations for human health or aquatic toxicity [4]. For example, hazardous waste sites potentially exert adverse effects on terrestrial ecosystems, and plant populations and communities may reflect those adverse effects. Plants may be evaluated in both the laboratory and the field [5, 6], and contribute to hazardous waste site evaluations. Within laboratory settings,

plant toxicity assessment includes methods which assess critical developmental stages in plant life history [7] and yield information pertinent to the evaluation of ecological effects in the field [2]. The seed germination and root elongation assays are both derived from tests traditionally used in plant science, as well as the fertilizer and herbicide evaluation [e.g., 8 - 14]. In this paper we will review these two phytotoxicity test methods, consider the development of the root elongation toxicity test and its miniaturization to yield a cost-effective tool for toxicity assessment, and illustrate how the seed germination test may contribute to the toxicity assessment process for a waste site.

## Test Matrices for Phytotoxicity Assessments

Within laboratory settings, site-soils must be managed to maintain their integrity so they remain representative of the site being evaluated [2]. At the same time, site-samples must be amenable to laboratory toxicity test conditions and conform to the specifications of standard test methods. Samples obtained from hazardous waste sites frequently represent heterogeneous mixtures of anthropogenic chemicals and chemicals which occur naturally in the substrate (e.g., clays, silts and sands in varying proportions) [15, 16, 17]. Field sampling of site-soils may be considered the most critical step in soil toxicity assessment, particularly if those assessments are derived from

toxicity testing completed at off-site laboratories [2]. Furthermore, to establish linkages between chemicals present on the site and biological effects noted in the toxicity assessment, soil samples collected for plant toxicity testing should be compatible with sample requirements for soil chemical analyses.

## Critical life stage toxicity assessment methods using plants

**Seed germination (120-hour) toxicity test.** The seed germination test yields toxicity information which reflects the potential hazard associated with soil directly [2]. Seed germination represents a critical stage in the developmental biology of plants [18], and the integrative responses associated with exposures of seeds to site-soil contaminants may yield toxicity information pertinent to site evaluation and characterization. The seed germination test summarized here [7] represents a modification of a method developed by Thomas and Cline [19].

**Site-soil preparation and toxicity testing.** Toxicity tests like the seed germination bioassay are performed directly upon site-soils and require site-soil samples being mixed with artificial soil to derive test concentrations. Depending upon the site-specific requirements, exposure series may be adjusted for pH and hydration, and cover a logarithmically spaced series of test concentrations. Test soils are loaded into Petri dish bottoms to yield a range of exposure concentrations (e.g., 80%, 40%, 20%,

10%, 5%, and 0% site-sample mixed with artificial soil). Forty seeds (seeds of commercially important species are commonly tested) are planted onto each replicate test soil, then covered with 16-mesh silica sand and irrigated to a nominal 85% water holding capacity. The loaded Petri dish is incubated in a sealed plastic bag, with as much air space as possible left inside the closed system (Figure 1 after Thomas and Cline [19]). Incubation occurs in a growth chamber for 120 hours (24 +/- 2°C); the first 48 hours are completed in total darkness and the balance of the incubation occurs at 16:8 light:dark. During the last 72 hours the photophase characteristically occurs at 4304 +/- 430 lux. After 120 hours the number of seeds which have germinated are counted and toxicity estimates ($LC_{50}$s) are calculated using the trimmed Spearman-Karber method [20] or other appropriate statistics of bioassay [e.g., 21 - 24].

**Root elongation (120-hour) toxicity test.** Unlike the Neubauer seed germination toxicity test, the short-term (120-hour) root elongation bioassay evaluates only the water-soluble constituents of a site-soil eluate and is completed without site-soils being included in the test system [2]. Depending upon the water solubility of soil contaminants, root elongation is generally inhibited at lower toxicant concentrations than is seed germination [7, 25 - 28]; hence, root elongation may be a more sensitive indicator of biological effects. As with the seed germination toxicity test, root elongation tests may be performed

with any number of species which represent economically important plants that are readily available, germinate and grow rapidly [29].

**Soil eluate preparation and toxicity testing.** Indirect tests which evaluate the mobility of chemical constituents characteristic of hazardous waste site complex mixtures may be completed with eluates prepared from untreated site-soils. Exposures in these indirect toxicity bioassays reflect percent eluate mixed with dilution water, and are performed using a logarithmically spaced series of concentrations capable of yielding the desired toxicity measures [7]. The test is performed using graded seeds which are placed in filter paper-lined Petri dish exposure chambers. In addition to deionized water controls, a logarithmic series of test concentrations (volume:volume percent) is prepared and added to the filter paper-lined Petri dish bottoms. Then, test seeds are spaced equidistantly on the saturated filter paper. The covered Petri dishes are incubated in a darkened, humid container for 120 hours at 24 +/- 2°C. At the end of 120-hours, root length is measured. While morphological changes in roots may suggest potential toxic effects associated with exposure, the measurement endpoint in these tests is linear growth; root elongation is measured along the primary growth axis (manually [see Figure 3] or with planimeters or root area meters) and effective concentrations which yield 50% inhibition in root elongation ($EC_{50}$s) are

calculated according to statistical procedures outlined in Stephan [30] and Stephan and Rogers [31].

**Root elongation toxicity test development and validation**

The root elongation toxicity test outlined above exemplifies the development of tools applicable to toxicity assessment. As a modification of the method used by Vandecaveye [14], the development of the root elongation toxicity test required comparisons to previously completed interlaboratory tests as an indirect evaluation of the method's ruggedness. Ratsch [32] in an interlaboratory test followed a "larger-scale" procedure originally developed by industry [33] as part of their compliance with the Toxic Substances Control Act (TSCA). In the "larger-scale" procedure, tested seeds were "planted" on glass plates which supported chromatography filter paper substrates saturated with test solution. These glass plates were placed into molded glass incubation chambers containing one liter of test solution which assured that the bottom 2 cm of the filter paper-covered plate was immersed in the test solution (Figure 2 after Ratsch [32]). Incubation conditions and measurements of root growth (root lengths measured from the transition point between the hypocotyl and root to the tip of the root; see Figure 3 after Ratsch [32]) were similar to those in the Petri dish method summarized above.

The "scaled-down," Petri dish technique reduces test solution volumes, and also reduces the cost associated with test materials and their disposal upon completion of the test. A comparison between the "larger-scale" test system [32] and the "scaled-down," Petri dish modification [7] illustrates the comparability in root elongation test results generated by each method for a selected group of chemicals. In Table 1, a summary of pertinent toxicity endpoint comparisons is illustrated. On the basis of effective concentrations which yield 50% reductions in root elongation ($EC_{50}$s), the two methods presented no statistically significant differences among chemical rankings nor among species responses to the test chemicals (Mann-Whitney test, P = 0.05 [34]). The application of the modified test to phytotoxicity assessment then finds support from these comparative analyses; the "scaled-down," Petri dish modification yields results not unlike those generated by the "larger-scale" method used by Ratsch [32] earlier. For example, each method suggested a similar ranking of species sensitivities, and presented a highly correlated ranking (Spearman rank correlation, P = 0.05 [34]) of test chemicals when these toxicity tests were used to compare inhibition of root elongation (Table 1). While quantitative differences in their measurements of root elongation were apparent (e.g., $EC_{50}$ estimates derived from "scaled-down" method were generally higher than those derived from the "larger-scale" method; see Table 1), those differences were not surprising in view of each method's unique exposure system. Furthermore, the

measurements of root elongation in the modified, "scaled-down" technique reflected test species variability which was not inconsistent with the findings of Ratsch [32] in his initial interlaboratory comparison completed with the "larger-scale" test system.

## Seed germination tests and waste site toxicity assessments

As illustrated by the root elongation toxicity test, cost-effectiveness may drive toxicity test development and modification, particularly when phytotoxicity assessments at hazardous waste sites are being developed. Similarly, ecological assessments for hazardous waste sites may encourage additional modifications and applications for phytotoxicity tests throughout site characterization and evaluation [5]. For example, toxicity estimates for a site-soil may be derived from either seed germination or root elongation tests and may be used to improve site evaluations. Toxicity-based criteria then may be integrated into management decisions regarding the disposition of the site [6]. Such toxicity-based guidelines potentially complement hazard assessment strategies formerly reliant chiefly upon site-sample chemical analyses; for most hazardous waste sites, toxicity-based criteria have become increasingly important owing to the complex chemical mixtures characteristic of these sites. Phytotoxicity assessment tools may also suggest the efficacy of proposed remediation methods for site clean-up. Within the

context of site characterization and evaluation, recent studies [35] illustrate the role which seed germination tests may contribute to the process.

The Bunker Hill Superfund site located in the Silver Valley of Idaho was listed as a priority site for clean-up by the U.S. Environmental Protection Agency in 1982 under the authority of the Comprehensive Environmental Response, Compensation, and Liability Act of 1980 (CERCLA). Being a mine tailings waste site, Bunker Hill presented high concentrations of heavy metals in site-soils, and the chief concerns were contaminants like lead, cadmium, and zinc [35]; preliminary chemical analysis determined that these chemical constituents were grossly elevated in the contaminant mixture present in the site-soil (Table 2). As a result of a preliminary field survey which identified altered vegetative characteristics at the site, both laboratory and greenhouse phytotoxicity assessments were included as part of the site evaluation and were completed with site-soil samples. Preliminary phytotoxicity assessments completed in the laboratory included the modified Neubauer seed germination toxicity test. With both site-samples and site-samples mixed with proposed soil amendments (zeolites were identified as additives potentially capable of sequestering heavy metal contaminants), preliminary screening tests adapted from the seed germination toxicity test [7] were completed in conjunction with greenhouse exposures. A summary of these laboratory-generated phytotoxicity measurements

is included in Table 3, which also includes brief summaries of the greenhouse potted-plant toxicity assessments which were completed with the same site-samples [35]. From the greenhouse experiments completed by Krawczyk, et al. [35], germination was variable for seeds (bush bean, Phaseolus vulgaris L. var. Blue Lake 290 and tall fescue, Festuca arundinacea Shreb) exposed to test soils, and quantitative comparisons were not possible because of variable environmental conditions at the soil surface. The complementary phytotoxicity assessment using lettuce seed in the modified Neubauer seed germination toxicity test, however, indicated a zeolite concentration-dependent reduction in test seed germination, which suggested that critical early developmental effects would remain, even in amended site-soils. Indeed, as suggested by the biomass data in Table 3, Krawczyk, et al. [35] found that bean and fescue showed decreased yields in amended soils, and that site-soils amended with zeolites were no less toxic to plants than the unamended soil. As evident from these results, the two assessment tools were complementary, and suggested that the proposed amendments may not significantly diminish the phytotoxicity associated with the Bunker Hill soils [35]. Additionally, these toxicity assessments corroborated the preliminary biological survey completed at the site, and suggested additional phytotoxicity assessment work may be a valuable contribution to future ecological evaluations for the site.

## Phytotoxicity Tests and Ecological Assessments at Waste Sites

Within the ecological assessment process, both the seed germination and root elongation phytotoxicity tests may contribute to hazardous waste site evaluations [6, 36, 37]. As previously suggested [5], toxicity assessments may be valued components in the site characterization, evaluation, remediation, or monitoring process, and if considered alone, the seed germination and root elongation tests may directly contribute to an assessment of the phytotoxicity associated with hazardous waste sites. But, if regarded within an integrated laboratory and field assessment strategy, their contributions as measurements of toxicity may suggest the possible ecological consequences potentially associated with exposures in the field, as well as more firmly establish linkages between site-specific chemical constituents and the biological responses characteristic of the site. Thomas, et al. [38] suggested, for example, that laboratory toxicity assessments may contribute to waste site evaluations. By applying spatial statistic techniques such as kriging to endpoints derived from the lettuce seed germination test, Thomas et al. [38] "mapped" toxicity data and suggested that such methods could potentially contribute to site clean up recommendations. Similarly, the brief consideration of the integrated laboratory-greenhouse evaluations completed for the Bunker Hill site suggested that these more controlled laboratory exposure systems may complement field surveys and contribute to

site remediation decisions. As illustrated here, the potential role of laboratory methods in site remediation could provide pertinent information regarding the potential effectiveness of various remedial actions.

Considering the demands placed upon phytotoxicity test systems, methods development has been responsive to a variety of problems, and limitations in sample volumes has frequently been a consideration for hazardous waste site toxicity assessments. As suggested by the development of the root elongation test outlined here, method modifications and refinements allow reduced site-sample volumes for testing, yet yield toxicity endpoint estimates not unlike those obtained from techniques which require larger sample volumes. The root elongation method summarized here correlates well with other test methods used in phytotoxicity assessments [39 - 43]; these phytotoxicity test methods differ with respect to quantitative estimates of their common toxicity endpoint, root elongation, but as assessment tools, they yield quite similar rankings for chemicals tested to date [39]. The root elongation test as well as the modified Neubauer seed germination test minimize the test sample volumes required, but the data base currently available suggests the toxicity endpoints relevant to site evaluation are not unduly compromised. The root elongation and seed germination methods summarized here, however, do reflect work which has been completed primarily with reference toxicants in single-compound exposures, and work with defined

chemical mixtures and complex chemical mixtures from waste sites is needed in order to fully describe the limitations as well as robust characteristics of these phytotoxicity test systems.

## Conclusions

Phytotoxicity assessments are playing a critical role in the evaluation of adverse ecological effects at hazardous waste sites, and plant toxicity tests which are amenable to the task have been developed or modified in response to this increasing demand. Seed germination is a critical stage in plant life history as is root elongation, and both biological events have been evaluated as toxicity endpoints relevant to phytotoxicity assessment. As derivatives from plant and weed science, the seed germination and root elongation tests have both undergone modifications which have increased their applicability to toxicity assessment. Reductions to minimize testing volumes have been central to methods development, and have yielded techniques which measure the toxicity of soils or soil-sample derivatives such as eluates and leachates, while minimizing the amount of material required for testing and subsequent disposal. Similarly, plant toxicity assessment methods have gained increased applications in waste site evaluations, and have been used during all phases of site assessment from site characterization through site remediation and monitoring. *In situ* methods which complement these techniques for laboratory

phytotoxicity assessment are currently being developed, and the integration of field assessment tools with these techniques will enhance their contribution to ecological assessments for hazardous waste sites.

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Figure 1. After Thomas and Cline [19].

Figure 2. After Ratsch [32].

Figure 3. After Ratsch [32]. Arrow indicates transition point between hypocotyl and root used in measuring root length (transition point to root tip).

Table 1.

COMPARISON OF ROOT ELONGATION TOXICITY TEST ENDPOINTS. Ratsch [3%] and Greene, et al. [7] methods used to evaluate biological effects of reference toxicants.

| TOXICANT1 | CLOVER2 | | LETTUCE2 | | WHEAT2 | |
|---|---|---|---|---|---|---|
| | "scaled-down" | "larger-scale" | "scaled-down" | "larger-scale" | "scaled-down" | "larger-scale" |
| CdCl2 | 29.3 (4.7) | 18.4 (4.1) | 10.5 (1.4) | 8.6 (1.9) | 35.9 (7.2) | 99.7 (17.9) |
| AgNO3 | 131.2 (16.6) | 124.7 (35.9) | 21.9 (3.0) | 3.3 (1.0) | 87.4 (23.5) | 167.7 (28.0) |
| NaF | 277.6 (27.7) | 311.6 (29.3) | 467.8 (41.3) | 496.7 (34.4) | 501.9 (79.3) | 238.1 (13.1) |
| MA | 32.1 (8.4) | 18.9 (2.3) | 36.6 (4.5) | 20.3 (3.6) | 405.2 (143.1) | 681.3 (204.5) |
| 2,4-D | 0.16 (0.11) | 0.053 (0.014) | 0.019 (0.005) | 0.043 (0.009) | 0.34 (0.068) | 3.75 (1.31) |
| Monuron | 155.0 (18.4) | 64.6 (11.5) | 54.6 (9.5) | 83.4 (12.4) | 40.8 (10.1) | 179.5 (19.2) |

| TOXICANT1 | CUCUMBER2 | | RADISH2 | | RANK SCORE (+/- 1 S.D.) | | ROOT ELONGATION |
|---|---|---|---|---|---|---|---|
| | "scaled-down" | "larger-scale" | "scaled-down" | "larger-scale" | "scaled-down" | "larger-scale" | "scaled-down" > "larger-scale" |
| CdCl2 | 41.5 (5.5) | 19.7 (3.5) | 153.0 (24.6) | 18.9 (3.5) | 2.4 (0.9) | 2.2 (0.4) | 4 > 1 |
| AgNO3 | 105.5 (10.9) | 27.4 (9.1) | 275.2 (37.1) | 73.9 (13.6) | 3.8 (0.8) | 3.4 (1.1) | 4 > 1 |
| NaF | 227.9 (38.6) | 166.4 (10.1) | 412.5 (29.6) | 354.3 (34.5) | 6.0 (NC) | 5.8 (0.4) | 3 > 2 |
| MA | 146.3 (23.8) | 92.9 (12.3) | 87.1 (47.6) | 28.7 (6.2) | 3.8 (1.3) | 4.2 (1.3) | 4 > 1 |
| 2,4-D | 0.074 (0.010) | 0.045 (0.019) | 0.065 (0.010) | 0.038 (0.006) | 1.0 (NC) | 1.0 (NC) | 3 > 2 |
| Monuron | 136.2 (19.7) | 89.9 (17.0) | 151.4 (16.8) | 99.6 (19.0) | 4.0 (1.0) | 4.4 (0.5) | 3 > 2 |

1 toxicant concentrations in mg/L (as [Cd]; [Ag]; [F], [methyl arsonate]; [2,4 - D]; [Monuron]; MA = methanearsonic acid.
2 EC50 expressed as toxicant concentration associated with 50% reduction in root length after 120 hours.

Table 2. Bulk chemical analysis of unamended Bunker Hill site soil major and minor constituents (after Krawczyk, et al. [32]).[1]

| Major constituents (percent concentrations) | | Minor constituents (mg/kg) | |
|---|---|---|---|
| Fe | 14.1 ± 1.3 | Cu | 483 ± 42 |
| Mn | 1.4 ± 0.1 | Cd | 65 ± 7 |
| Pb | 2.2 ± 0.2 | P | 314 ± 27 |
| S | 0.8 ± 0.1 | Na | 256 ± 109 |
| Zn | 0.9 ± 0.1 | Sr | 8.5 ± 0.6 |

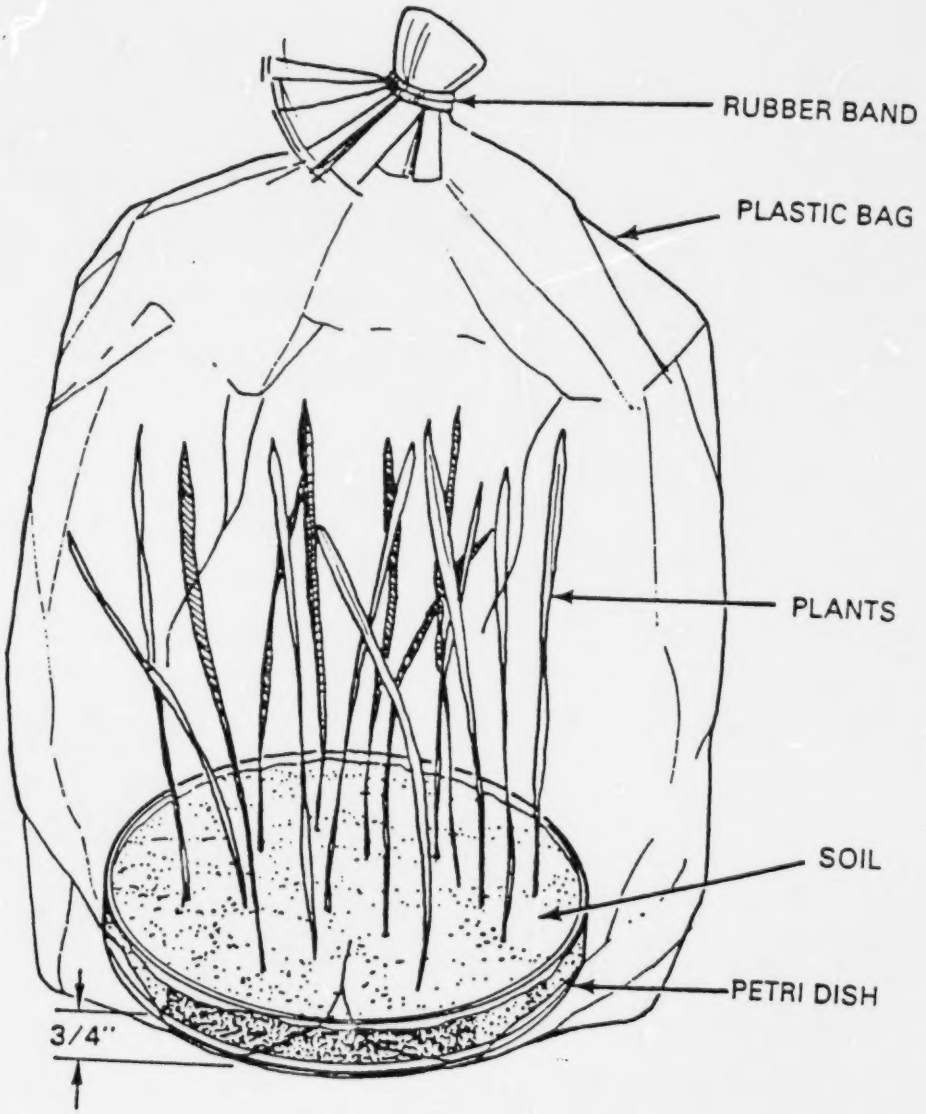
[1] Analyses completed using U.S. Environmental Protection Agency Methods 3050 and 6010, mean (± S.D.) of triplicate determinations.

Table 3. Summary data sheet for toxicity assessments on Bunker Hill site-samples completed using modified Neubauer toxicity test and greenhouse potted-plant exposures.

| Site-sample | Seed [1] Germination | Total Biomass [2] Bean | Fescue |
|---|---|---|---|
| Zeolite, Bunker Hill (unamended) | 95 ± 5 | 1.0 | 0.8 |
| Zeolite, Bunker Hill (limestone amendment) | 93 ± 4 | 4.0 | 0.4 |
| Zeolite, Bunker Hill (XY 5, 4% amendment) | 90 ± 11 | 0.6 | 1.1 |
| Zeolite, Bunker Hill (XY 5, 12% amendment) | 77 ± 2 | 1.2 | 0.7 |
| Zeolite, Bunker Hill (XY 5, 20% amendment) | 40 ± 4 | 1.3 | 1.2 |
| Zeolite, Bunker Hill (CH 20, 4% amendment) | 90 ± 2 | 1.4 | 0.7 |
| Zeolite, Bunker Hill (CH 20, 12% amendment) | 73 ± 10 | 0.8 | 0.8 |
| Zeolite, Bunker Hill (CH 20, 20% amendment) | 62 ± 4 | 1.1 | 0.7 |
| Control Soils | | | |
| Hyslop control | N.T. | 12.0 | 11.0 |
| Germination control [3] | 95 ± 5 [4] | 26.0 | 18.0 |
| Greenhouse control | N.T. | 32.0 | 16.0 |

[1] mean (± S.D.) percent lettuce seed germination at 100% site-sample, n = 3.
[2] modified from Krawczyk, et al. [32]; mean dry weight/plant after 60 days growth.
[3] Germination control consists of 70% sand, 20% kaolin, 10% screened peat, and 0.42% $CaCO_3$.
[4] data base developed in support of Greene, et al. [7] using Thomas and Cline method [19] with sand as control matrix.

1 Figure 1. After Thomas and Cline [19].



1 Figure 2. After Ratsch [32].

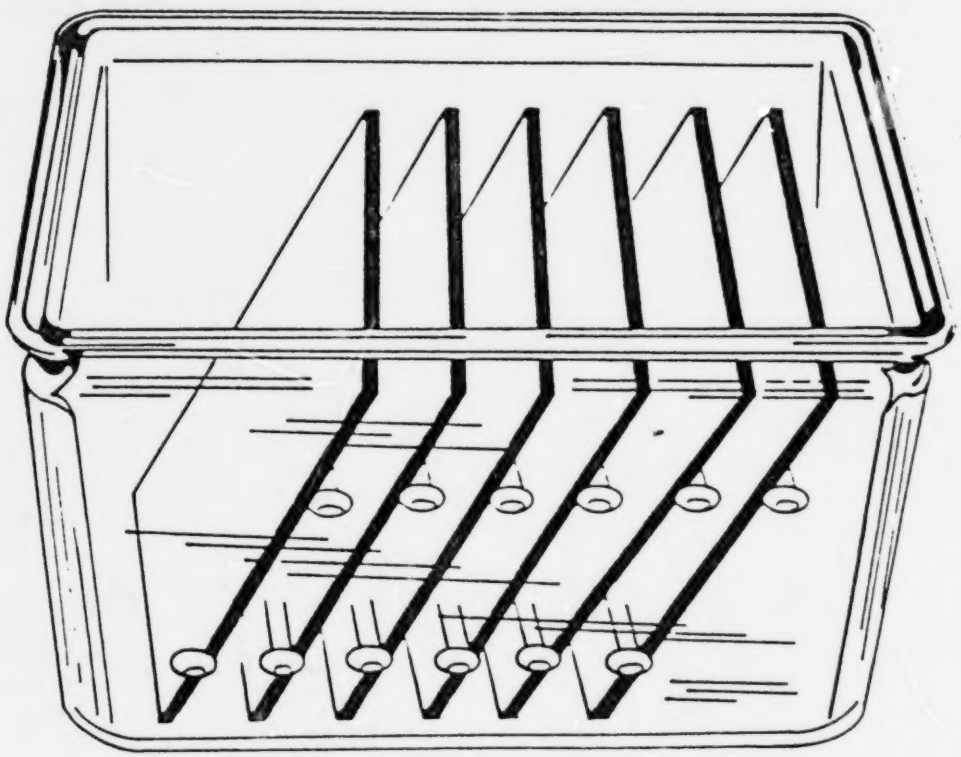


Figure 3. After Ratsch [32]. Arrow indicates transition point between hypocotyl and root used in measuring root length (transition point to root tip).